THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

The usefulness and reliability of the receptorial responsiveness method (RRM), with special regard to the influence of properties of agonists and the biological system

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The Examination takes place at the Library of the Department of Pharmacology, Faculty of Pharmacy, Medical and Health Science Center, University of Debrecen
11 h, May 12, 2011

Head of the Defense Committee: Prof. Dr. Lajos Gergely

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute of Internal Medicine, Medical and Health Science Center, University of Debrecen
13 h, May 12, 2011

1. Introduction

In the present PhD thesis, results of *ex vivo* and *in silico* investigations are presented. All investigations are strongly associated with a recently developed procedure, the so-called receptorial responsiveness method (RRM), a special non-linear regression algorithm capable of determining an increase in the near-receptor concentration of a pharmacological agonist.

Functions of multi-cell organisms (possessing a “milieu interieur”) are influenced by several endogenous regulatory molecules, most of which can be considered as a receptor agonist. The link between an agonist concentration and its effect can be most precisely determined by constructing a concentration-effect (E/c) curve and then by fitting it to an appropriate equation (e.g. see: equations 3.2. and 3.3. on p. 11).

Usually, when analyzing an E/c curve, stimulation of the receptors is ascribed exclusively to the exogenous agonist that is administered for the E/c curve. If there is some endogenous agonist at the receptors, this is typically neglected during the evaluation. However, when the system contains a surplus agonist in a concentration that is sufficient to exert significant receptor stimulation (and thereby it is able to decrease considerably the response capacity of the receptors), the subsequently generated E/c curve will be obviously biased. Our work group previously described the relationship between the concentration of this surplus agonist (referred to as “biasing agonist”) and the bias of the E/c curve constructed with a so-called “test” agonist in the given system (see: equation 3.8. on p. 13).

Although the simplest case is when the biasing and the test agonist are the same, but this is not essential to achieve a useful result. This is due to the fact that even stimulatory effects of different agonists cumulate, at least in part, on the same structures (receptors, postreceptorial signaling elements), “response

capacity” of which is also limited. So, it seems to be enough if the test agonist act on the same receptor as the biasing agonist, or it influences a postreceptorial signaling largely overlapping with that of the biasing agonist. In order to handle the simultaneous action of two (possibly different) agonists the simplest way, the relationship between biasing concentration and E/c curve bias was derived by replacing the biasing agonist concentration (c_{bias}) with the equieffective concentration of the test agonist (c_x).

Based on the above-mentioned relationship, an unknown concentration of a biasing agonist can be estimated from the bias of the E/c curve. If an endogenous agonist is released (or synthesized) in the microenvironment of its receptor, or has a short half-life, determination of its effective concentration is hard to perform. However, if we can generate two E/c curves in the given system: an E/c curve before and another one after the release (or synthesis) of this agonist, the difference between these E/c curves (i.e. the bias of the second curve) will hold information about the concentration of the endogenous agonist in question. This information can be elicited by fitting data of the secondly constructed (biased) E/c curve to the above-mentioned relationship (between biasing concentration and E/c curve bias), which is expressed as a function of the biased effect (an effect computed ignoring the biasing agonist) versus the concentration of the test agonist (see: equation 3.8. on p. 13). This function should contain the relevant parameters of the actual (unbiased) link between the concentration of the test agonist and its effect in the given system (when using the Hill model, these parameters are E_{max}, EC_{50} and n; for explanation see: equation 3.2. on p. 11). The source of these parameters is the firstly generated (and thereby unbiased) E/c curve. Of course, this means that, in reality, the estimate provided by this method is the change in concentration of the biasing agonist in the system between constructions of the two E/c curves. This procedure, named RRM, implies some limitations, such as receptors should not

desensitize during the assessment, and the biasing agonist concentration should be constant under the construction of both E/c curve.

If the biasing and the test agonist are the same, the result directly estimates the biasing agonist concentration in question. If not, the RRM provides a test agonist concentration that is equieffective with the biasing agonist concentration. The use of a stable compound as a test agonist greatly increases precision and accuracy of the E/c curve. Thus, allowing the biasing and the test agonist to differ, the RRM appears to be suitable to gather useful data about changes in concentration of degradable endogenous agonists at their receptors, a tissue compartment otherwise difficult to access.

However, if the biasing and the test agonist, being different, possess dissimilar properties such as affinity and efficacy, theoretical concerns may emerge regarding the reliability of estimates obtained by the RRM. Addressing this issue may have practical significance, with regard to the promising ability of the RRM to estimate changes in concentration of degradable agonists in functioning tissues.

During the investigations that form the basis for the present PhD thesis, therefore, theoretical E/c curves were constructed that simulated the co-action of pairs of agonists with different properties. One member of these agonist pairs was used at a fixed concentration as a biasing agonist, and the other one at variable concentration as a test agonist. Then, these E/c curves were transformed into a form as if the biasing concentration had been neglected during their construction. Afterward, the biasing agonist concentrations were estimated using the RRM from the transformed (and thereby “biased”) E/c curves and from E/c curves simulated the effect of the given test agonist alone (unbiased curves). The estimates were compared to the input values (c_{bias}) in order to assess the reliability of the RRM in case of dissimilar biasing and test agonists. To investigate the influence of the pharmacological system on the results obtained by the RRM, E/c curves mentioned above were generated with different slope

factors. In addition, the above-mentioned procedure was repeated out using another equation for RRM. This newly constructed equation incorporated the Richards equation, which can handle asymmetric E/c curves, instead of the Hill equation, which defines exclusively symmetric functions (see: equations 3.2. and 3.3. on p. 11). Accuracy of estimates of biasing agonist concentrations provided by these two different equations of RRM were compared to assess whether allowance of asymmetry of E/c curves influences the reliability of RRM.

During the *ex vivo* and *in silico* investigations of the present PhD thesis, the following four questions were aimed to address:

1. How does the inhibition of adenosine deaminase (ADA) influence the effect of an ADA-resistant A_1 receptor agonist in eu- and hyperthyroid guinea pig left atria? Analysis of this problem requires the consideration of principles of RRM.

2. How does the magnitude of c_{bias} and operational slope factor (n_{op}), a parameter defined by the operational model of agonist action to characterize the pharmacological system, influence the accuracy of RRM (when the biasing and test agonist is the same)?

3. How does affinity and efficacy of the agonists influence the accuracy of RRM if the biasing and test agonist are different?

4. How does allowance of asymmetry of E/c curves influence the accuracy of RRM?

2. *Ex vivo* methods

2.1. Solutions

The following materials were used: salts for the modified Krebs-Henseleit buffer (Krebs buffer); adenosine; N^6-cyclopentyladenosine (CPA); *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA); 2'-deoxycoformycin (pentostatin; DCF); L-thyroxin (T_4). The bathing medium for the preparations was Krebs buffer (36 °C) in the entire course of the experiments.

2.2. *In vivo* treatment

Our experiments were performed on the isolated left atria of male guinea pigs weighing 400-700 g. The housing, pretreatment and processing of animals was according to the European Community guidelines and in agreement with the Ethical Codex of the Committee of Experimental Animal Research (University of Debrecen).

The animals were randomly divided into two groups: the T_4 treated and the solvent (S) treated group. One group of the animals received 330 μg/kg L-thyroxin sodium salt pentahydrate (T_4) daily ip. for 8 days (in vivo T_4 treatment), while the vehicle of T_4 (S) was administered daily ip. for 8 days to another group (in vivo solvent treatment). The animals were sacrificed on the ninth day by one firm blow on the head.

2.3. Tissue preparations and pre-incubation

After opening the thorax of the guillotined guinea pigs, left atria were removed and mounted in a 10 cm3 organ chamber filled with Krebs solution

(TSZ-04, Experimetria, Budapest). The Krebs solution was oxygenated with 95 % O_2 and 5% CO_2 (pH 7.4). Atria were fixed to an isometric transducer (SG-01 D, Experimetria, Budapest) under a tension of 10 nM. Atria were stimulated by a programmable electrical stimulator (ST-02, Experimetria, Budapest) through platinum electrodes. Stimulation was performed at a frequency of 3 Hz with 1 ms impulse width and under 150 % threshold tension (approximately 1 V) the electrical signs were registered with a 6 channel polygraph (BR-61, Medicor, Budapest).

After starting the stimulation, every atrium was incubated in a Krebs solution for 50 minutes to get the contractility parameters stabilized. The bathing medium was changed every 15 to 20 minutes (washing). Atria in the S and T_4 treated groups were randomized in 4-4 subgroups during pre-incubation (see below). During the experiments the isometric contractions of the atria were registered and the amplitudes of these contractions were assessed as contraction force.

2.4. Adenosine E/c curves

First in all experimental subgroups, a cumulative E/c curve was generated with adenosine to validate the thyroid status. Due to its short half-life, adenosine used did not influence the subsequent E/c curve.

2.5. *In vitro* treatment

After the wash-out of adenosine, one of two protocols were carried out. The longer protocol contained an in vitro treatment (60 min.) for the following subgroups:

1. S treated control subgroup (S Co; n = 7)

2. S and EHNA treated subgroup (S EHNA; n = 7)

3. S and DCF treated subgroup (S DCF; n = 8)

4. T_4 treated control subgroup (T Co; n = 7)

5. T_4 and EHNA treated subgroup (T EHNA; n = 8)

6. T_4 and DCF treated subgroup (T DCF; n = 8).

The other protocol consisted of a 15-20 min. wash-out for the following subgroups:

1. S treated subgroup (S Ø; n = 4)

2. T_4 treated subgroup (T Ø; n = 4).

2.6. CPA E/c curves

In the *in vitro* treated subgroups, a cumulative E/c curve was constructed with CPA, a selective A_1 receptor agonist. Because CPA is not a substrate for the adenosine-handling enzymes present in the asanguineous myocardium, its concentration scarcely diminished during our experiment (approximately 20-40 minutes). Consequently, CPA can be hardly removed from the atrium. For this reason, we did not perform self controlling experiments, rather we created distinct control subgroups.

2.7. Inosine E/c curves

In the two subgroups without *in vitro* treatment, a cumulative E/c curve with inosine was constructed. In some experimental models, inosine was found to affect the adenosinergic mechanisms of the heart, so we investigated its effect on the atrial contractility in our model.

2.8. Evaluation of the contractile forces and E/c curves

The percentage decrease in the initial contraction force was considered as the effect of the given agonist concentration. The E/c curve was fitted to the Hill equation to yield the E_{max}, EC_{50} and n parameters characterizing the E/c curves.

2.9. Statistical analysis

For the comparison of the average values of two groups with normal distribution and homogenous variations, two-sided, two-sample, unmatched Student t-test was carried out. In the case of Gaussian distribution and differing variations Welch's t-test was chosen. For more than two groups following Gaussian distribution, one-way ANOVA with Newman-Keuls post-testing was performed.

To compare E/c curves, the averaged effect values as well as the E_{max}, $\log EC_{50}$ and n values provided by the fitted Hill equation were used.

3. *In silico* methods

E/c curves were constructed that simulated the co-action of pairs of agonists with different properties. One member of these agonist pairs was used at a fixed concentration as a biasing agonist, and the other one at variable concentration as a test agonist. Then, these E/c curves were transformed into a form as if the biasing concentration had been neglected during their construction. Afterward, the biasing agonist concentrations were estimated using the RRM from the transformed (and thereby "biased") E/c curves and from E/c curves simulated the effect of the given test agonist alone (unbiased curves). In some cases, to assess the influence of E/c curve asymmetry on the accuracy of the RRM, it was used in two forms, i.e. two equations were fitted: one containing the Hill equation and another one incorporating the Richards equation. Finally, the estimates were compared to the input values. In addition, to investigate the influence of the pharmacological system on the results of RRM, E/c curves mentioned above were generated with different slope factors.

3.1. Construction of unbiased E/c curves with one agonist

Theoretical agonists and systems were defined in terms of the operational model of agonism. Using parameters of these agonists and systems, effect values were computed with the basal equation of the operational model:

$$E = E_m \cdot \frac{[R_0]^{n_{op}} \cdot c^{n_{op}}}{K_E{}^{n_{op}} \cdot (K + c)^{n_{op}} + [R_0]^{n_{op}} \cdot c^{n_{op}}} \qquad 3.1.$$

where: E is the effect; E_m is the possible maximum effect in the system; $[R_0]$ is the receptor concentration; c is the concentration of the given agonist; K

is the equilibrium dissociation constant of the agonist-receptor complex (a measure for agonist affinity); K_E is a measure of agonist efficacy; n_{op} is the operational slope factor.

Effects computed with the equation 3.1. were plotted versus the relevant agonist concentrations. The obtained functions can be considered as “unbiased” E/c curves of the agonists acting alone.

3.2. Fitting of the unbiased E/c curves with one agonist to the Hill or Richards equation

The unbiased E/c curves of agonists A-G (constructed in all the three systems) were fitted to both the Hill (3.2.) and the Richards (3.3.) equations:

$$E = \frac{E_{\max}}{1+10^{n\cdot(\log EC_{50}-\log c)}} \quad \text{and} \quad E = \frac{E_{\max}}{\left(1+10^{n\cdot(\log X_b-\log c)}\right)^S} \qquad \text{3.2. and 3.3.}$$

where (the still undefined parameters): E_{max} is the maximum effect that can be elicited by the given agonist in the given system; EC_{50} is the midpoint location that indicates the agonist concentration producing half-maximum effect; n is the Hill slope factor; S is the symmetry parameter (if S differs from unity, the function will be asymmetric); X_b is a parameter determined by the midpoint and the point of inflexion of the function: $X_b = EC_{50} \cdot \left(2^{1/S} - 1\right)^{1/n}$.

Results of fitting of the Hill and the Richards model were compared by an F test. Best-fit values relating to the test agonist were used for the RRM.

3.3. Construction of unbiased E/c curves with two agonists

Simultaneous effects of the biasing and test agonist pairs were computed with the following equation:

$$E = E_m \cdot \frac{\left(\tau_{test} \cdot c_{test} \cdot K_{bias} + \tau_{bias} \cdot c_{bias} \cdot K_{test}\right)^{n_{op}}}{\left(c_{test} \cdot K_{bias} + K_{test} \cdot K_{bias} + c_{bias} \cdot K_{test}\right)^{n_{op}} + \left(\tau_{test} \cdot c_{test} \cdot K_{bias} + \tau_{bias} \cdot c_{bias} \cdot K_{test}\right)^{n_{op}}} \quad 3.4.$$

where (the still undefined parameters): c_{test} and cbias are concentrations of the test and the biasing agonist, respectively; K_{test} and K_{bias} are K values for the test and the biasing agonist, respectively; τ_{test} and τ_{bias} (defined as $[R_0]/K_{Etest}$ and $[R_0]/K_{Ebias}$, respectively) are operational efficacies for the test and the biasing agonist, respectively.

Starting from the operational model, EC_{50} values were calculated from the equation as follows:

$$EC_{50} = K \cdot \frac{1}{\left(2 + \tau^{n_{op}}\right)^{\frac{1}{n_{op}}} - 1} \quad 3.5.$$

Other ECF values were calculated from the appropriate EC_{50} and n_{op} with the use of the following equation:

$$EC_F = EC_{50} \cdot \left(\frac{F}{100 - F}\right)^{\frac{1}{n_{op}}} \quad 3.6.$$

If effects calculated by the equation 3.4. were plotted versus the sum of c_{test} and c_{bias}, the obtained functions would be the “unbiased” E/c curves of the two co-acting agonists.

3.4. Distortion of the unbiased E/c curves with two agonists

Effects computed with the equation 3.4. were transformed (“biased”) using the following equation:

$$E' = 100 - \frac{100 \cdot (100 - E)}{100 - E_{bias}} \qquad 3.7.$$

where: E’ is the biased effect; E is the (unbiased) effect provided by the equation 3.4.; E_{bias} is the effect of c_{bias} (computed with the equation 3.4. when c_{test} is zero but other parameters are the same as in the case of the corresponding E).

Thus, transformation by means of the equation 3.7. simulated that an unheeded c_{bias} is present in the system that evoked an unheeded E_{bias} effect. Consistently, biased effects calculated with the equation 3.7. were plotted only against c_{test} values that resulted in “biased” E/c curves (totally ignoring cbias and E_{bias}).

3.5. Fitting of the biased E/c curves with two agonists to the equations of the RRM

The biased E/c curves were fitted to the equations as follows:

$$E' = 100 - \frac{100 \cdot \left(100 - \frac{E_{\max}}{1 + 10^{n \cdot \left(\log EC_{50} - \log \left(10^{\log c_x} + 10^{\log c_{test}} \right) \right)}} \right)}{100 - \frac{E_{\max}}{1 + 10^{n \cdot (\log EC_{50} - \log c_x)}}} \qquad 3.8.$$

$$E' = 100 - \frac{100 \cdot \left(100 - \frac{E_{\max}}{\left(1 + 10^{n \cdot \left(\log X_b - \log \left(10^{\log c_x} + 10^{\log c_{test}} \right) \right)} \right)^S} \right)}{100 - \frac{E_{\max}}{\left(1 + 10^{n \cdot (\log X_b - \log c_x)} \right)^S}} \qquad 3.9.$$

where (the still undefined parameters): c_x is the test agonist concentration that is expected to be equieffective with c_{bias} (c_x is the only best-fit value provided by the equation 3.8. or 3.9.); E_{max}, $logEC_{50}$, n in the equation 3.8. and E_{max}, $logX_b$, n, S in the equation 3.9. are best-fit values yielded by fitting of the Hill and the Richards equation, respectively.

By means of the equation 3.1., c_x values were converted into estimates, i.e. biasing agonist concentrations being equieffective with c_{bias}. To express and compare the accuracy of concentration estimation using the two different equations, percentage deviation of estimates from the corresponding cbias values was calculated as follows: $((estimate\text{-}c_{bias})/c_{bias})*100\%$.

3.6. Computer simulation and data analysis

The y values and all non-fitted parameters of the curves were computed with Microsoft Excel 2003. For the curve fitting and statistical analysis, GraphPad Prism 4.03 for Windows was used.

4. Results

4.1. Effect of ADA inhibition on the A_1 receptor mediated negative inotropy in eu- and hyperthyroid guinea pig atria

Inosine scarcely influenced the contractile force in either the S or T_4 treated atria. In contrast, CPA concentration-dependently decreased the atrial contractile force. The T_4 treatment reduced the effect of CPA in all subgroups, when compared to their S treated counterparts.

The differences among the solvent treated subgroups failed to reach the level of statistical significance either in terms of regression parameters or individual responses. In contrast, T_4 treatment intensified the effects of EHNA and DCF: EHNA significantly increased the E_{max}, while DCF significantly decreased the $logEC_{50}$ (i.e. increased the potency). Although EHNA also lowered the $logEC_{50}$ and DCF also raised the E_{max} in the T_4 treated atria, these changes did not reach the level of statistical significance.

4.2. Effect of affinity, efficacy and operational slope factor on the accuracy of RRM

4.2.1. Reliability of RRM when the biasing and the test agonist are the same

In the system defined by n_{op}=0.5, the fitted equation 3.8. ran below the data points at lower and medium test agonist concentrations (c_{bias}), while it ran above them at higher c_{bias} values. So, running of the curve in relation to the data points can be summarized as “below then above”. At lower and medium c_{bias}

values, estimation was relatively accurate, but at higher c_{bias} values, the percentage deviation of the estimates from c_{bias} significantly exceeded 100%.

Fit of the equation 3.8. as well as estimation was the most accurate upon n_{op}=1. The fitted curve contained apparently all data points. The percentage deviation remained between 0% and 100% in the whole range of c_{bias} values; moreover, except for the two lowest c_{bias} values, it did not exceed 10%.

In the system with n_{op}=1.5, curve fitting failed at the two lowest c_{bias} values. Running of the fitted equation 3.8. was “above then below” type. On the other hand, estimation was almost as good as in case of n_{op}=1, the percentage deviation remained between -100% and 10%.

Interestingly, lower and higher c_{bias} values were underestimated in the system with n_{op}=1.5, while they were overestimated upon n_{op}=0.5 . At the same time, in case of n_{op}=1, only the lowest c_{bias} values were overestimated.

Precision of the curve fitting poorly correlated with the accuracy of the estimation.

4.2.2. Reliability of RRM when the biasing and the test agonist are dissimilar

Regarding their operational efficacy, agonists of this investigation could be grouped as follows: group A, B, C with $\tau = 10$; group D, F, H with $\tau = 1$; and group E, G, I with $\tau = 100$. The same biasing concentration in the same system could be characterized by the same c_x value for biasing agonists with equal efficacy, so affinity did not influence the c_x obtained. When converting this shared c_x to estimates, they only differed from each other in their orders of magnitude, consistent with the corresponding K_{bias} values.

To allow for τ of the test agonist (agonist A) as well, τ ratios were generated (τ_{bias}/τ_{test}). The best fit of the equation 3.8. as well as the most accurate estimation was observed when using agonists in the group A, B, C ($\tau_{bias}/\tau_{test} = 1$)

in the system with n_{op}=1 (percentage deviation was well within ±10%). When decreasing or increasing n_{op} in the group A, B, C, running of the fitted curve became “below then above” or “above then below” types, respectively, especially at higher c_{bias} values. Accordingly, estimation accuracy declined as compared to the case being n_{op}=1, but still remained relatively good (percentage deviation was within ±20% and ±10% upon n_{op}=0.5 and n_{op}=1.5, respectively).

In the group D, F, H (τ_{bias}/τ_{test} = 0.1) along with n_{op}=1, running of the fitted equation 3.8. showed an “above then below” character, which became more accentuated in the system with n_{op}=1.5 . Upon n_{op}=0.5, however, fit of the curve got better, the “above then below” character was revealed only at higher c_{bias} values. Consistently, major overestimation occurred in systems with n_{op}=1 and n_{op}=1.5, while only a moderate overestimation was found in case of n_{op}=0.5 (percentage deviation ranged between 10% and 100%). Upon coincidence of higher c_{bias} and larger n_{op}, no estimate could be calculated from c_x, because the effect related to c_x (E_x) exceeded the E_{max} of the simulated E/c curves.

In the group E, G, I (τ_{bias}/τ_{test} = 10), fit of the equation 3.8. was relatively correct upon n_{op}=1, the fitted curve shoved a “below then above” character only at higher c_{bias} values. Decrease of n_{op} worsened, while increase of n_{op} ameliorated (almost perfect fit) the fit of the equation 3.8., just contrary to the case of the group D, F, H. Estimation accuracy in the group E, G, I proved to be good, it ranged between -20% and 20%, -10% and 0%, furthermore -20% and 0% upon n_{op}=0.5, n_{op}=1 and n_{op}=1.5, respectively.

4.2.3. Influence of E/c curve asymmetry (and its respect in the RRM’s equation) on the reliability of RRM

If n_{op} = 1 (i.e. when agonists elicited symmetric E/c curves), the RRM using the Hill model and that applying the Richards model yielded equal estimates. When n_{op} = 0.5, the RRM including the Richards model provided

usually (although not always) more accurate estimates than the RRM integrating the Hill model. Interestingly, if n_{op} = 2, the RRM using the Richards equation mostly yielded similar or somewhat less accurate estimates as/than the RRM including the Hill equation. Taking all together, no fundamental difference was found between estimates provided by the RRM encompassing the Hill and the Richards model.

Determination of agonist D (τ_{bias}/τ_{test} = 1) in the system with n_{op} = 1 provided perfect estimates; their percentage deviation from the corresponding c_{bias} values was zero. In accordance with this, the fitted equation 3.8. and 3.9. visibly contained all data points of the biased E/c curves. In case of other agonists and/or other systems, accuracy of the estimation as well as precision of the fit proved usually worse.

Increase of the τ_{bias}/τ_{test} ratio pushed down the fitted equations at lower test agonist concentrations, while it pushed up the function at higher test agonist concentrations. In contrast to the τ_{bias}/τ_{test} ratio, an $n_{op} > 1$ (with τ_{bias}/τ_{test} = 1) made the function of the RRM "above then below" type, whereas if $n_{op} < 1$ (and τ_{bias}/τ_{test} = 1), the RRM's function turned "below then above" type, in case of using either the Hill or the Richards model. Effects of the τ_{bias}/τ_{test} ratio and n_{op} proved to be additive, so they could amplify or reduce influence of each other.

A "below then above" running of the RRM's function (caused by high τbias/τtest ratio and/or small nop) was associated with almost perfect estimation accuracy. However, an "above then below" type curve was linked to less accurate estimates, especially when the fit of the RRM's function to the data points was considerably wrong.

If the τ_{bias}/τ_{test} ratio and c_{bias} was high with a small n_{op}, no c_x value could be obtained due to the downhill running of the function. Use of the Richards model did not affect this problem at all.

5. Discussion

5.1. Interaction of ADA inhibitors and T_4 at the level of the adenosinergic system in the guinea pig left atrium

The *ex vivo* experiments showed that in the hyperthyroid guinea pig atrium, the combined ADA and cGMP-stimulated 3',5'-cyclic nucleotide phosphodiesterase (PDE2) inhibition elicited by EHNA augmented the E_{max} of CPA to a larger extent than selective ADA inhibition conferred by DCF did. This finding confirms the earlier result of our work group that PDE2 inhibition can increase the effect of CPA. Furthermore, DCF caused a small but significant rise in the potency of CPA (revealed in lesser EC_{50}) in the hyperthyroid guinea pig atrium. This result suggests that ADA inhibition can approximate the suppressed efficiency of hyperthyroid A_1 receptor signaling to the euthyroid level. Since extracellular adenosine accumulation did not enhance the A_1 receptor mediated negative inotropy in neither euthyroid nor hyperthyroid guinea pig atrial myocardium in an earlier study of our work group, effects of ADA inhibitors presented in this thesis are supposedly mediated by a rise in intracellular adenosine level.

5.2. Influence of agonist and system properties on the reliability of RRM

In the *in silico* investigations underlying the present thesis, effect of c_{bias} as well as n_{op} on the estimation accuracy of the RRM was firstly investigated under conditions where the biasing and the test agonist was the same. The c_x values estimated c_{bias} values with considerable accuracy in all three systems (defined by the three n_{op}), when c_{bias} was approximately within the range from

EC_{25} to EC_{75} of the agonist. Outside of this interval, estimation accuracy remained relatively good, when $n_{op}=1$, but it significantly worsened, if n_{op} was smaller or greater than unity. Thus, system properties manifested in the steepness of the E/c curve influence the reliability of the RRM, but this effect appears to be only significant at marginal concentrations.

Furthermore, we found that different efficacy (but not affinity) for the biasing and test agonist influences the accuracy of estimates provided by RRM. However, RRM may provide useful and reliable information, even when the biasing and the test agonist (which were herein assumed to bind to the same receptor) are different. Our present results may contribute to the successful use of RRM by recommending the following: (1) The c_x should be related to the medium section of the E/[A] curve of the test agonist. (2) It is worthwhile to choose a test agonist whose E_{max} close to that of the biasing agonist, because, in this case, if c_{bias} is localized in the medium section of the E/c curve (of the biasing agonist), c_x will also be localized in the medium section of the E/c curve (of the test agonist). (3) If the fitted equation 3.8. shows a remarkable "below then above" or "above then below" character, estimates should be treated with caution (especially in the latter case), furthermore, it is worthwhile to perform a new measurement with a test agonist having greater or smaller τ, respectively.

In addition, our results indicate that E/c curve asymmetry does not significantly influence the estimation accuracy of RRM and does not prevent the typical shortcomings of this method. Thus, the simpler form of the RRM (including the Hill model) seems to be sufficient for further studies. It was also found that coincidence of a high τ_{bias}/τ_{test} ratio, small n_{op} and high c_{bias} can lead to an inverse effect elicited by the test agonist that foils the estimation.

Taking all together, results of the present study suggest that the RRM may be a useful tool to gather information about concentrations (more precisely: changes in concentration from a basal level) of endogenous agonists at a special tissue compartment, the microenvironment of the relevant receptors.

6. Summary

The receptorial responsiveness method (RRM), a special non-linear regression algorithm capable of determining an increase in the near-receptor concentration of an agonist, was utilized and investigated.

1. The inhibition of adenosine deaminase (ADA) enhances the negative inotropic effect of CPA, an ADA-resistant A_1 receptor agonist, on the hyperthyroid guinea pig left atrium. This may be due to an increase in the signal amplification of the hyperthyroid A_1 receptor in response to ADA inhibition, and this might be mediated by the surplus intracellular adenosine content.

2. The estimation of an agonist concentration by RRM is the most accurate in the case of symmetric concentration-response curves ($n_{op} = 1$), but for agonist doses between EC_{25} and EC_{75} (in its own concentration-response curve of the agonist to be estimated), the estimation is acceptable using every investigated operational slope factor (n_{op}). If the agonist concentration to be estimated is high and $n_{op} < 1$, estimates become wrong.

3. While affinity of an agonist to be estimated with RRM does not affect the estimation accuracy, the efficacy value (characterizing the ability of the given agonist to generate an effect once bound) does. However, the running of the fitted equation of RRM provides useful information about the reliability of estimates. If the fitted equation of RRM has a considerable “below then above” or “above then below” character in relation to data points of an E/c curve, the estimates should be treated with caution.

4. In most cases, allowing for E/c curve asymmetry does not ameliorate substantially the accuracy of RRM, and it has no significant influence on the limitations of the method. Thus, there is no reason to exchange the simpler Hill model in RRM for a more complicated one to comply with a possible asymmetry of E/c curves.

Publications underlying the Ph.D. thesis

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Posters and abstracts

Gesztelyi R, Zsuga J, **Grenczer M**, Jakab A, Szabó G, Juhász B, Lekli I, Vecsernyés M, Tósaki Á. Az adenozin dezamináz pentostatin általi gátlása növeli az M_2 muszkarin receptorok ingerlésével kiváltható negatív inotróp választ tiroxinkezelt tengerimalacok bal pitvarán. MÉT LXXI. Vándorgyűlése, Pécs 2007, p. 161.

Gesztelyi R, Juhász B, Zsuga J, **Grenczer M**, Jakab A, Vecsernyés M, Tósaki Á. A tiroxinkezelés hatása az A_1 adenozin receptorok 8-cyclopentyl-1,3-dipropylxanthine (CPX) általi gátolhatóságára tengerimalac bal pitvaron. MÉT LXXII. Vándorgyűlése, Debrecen 2008, p. 202.

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